Summary......
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Carbon, nitrogen and sulphur metabolisms are highly dependent upon one another. Carbon metabolism is usually mediated by photosynthesis and respiration. Nitrogen metabolism is regulated via nitrate uptake by the root system and its reduction to \( \text{NH}_4^+ \). The S and N assimilation is mediated by a prosthetic group siroheme, synthesized from uroporphyrinogen III, an intermediate of biosynthetic pathway of Chl, an essential pigment of carbon assimilation in oxygenic prokaryotic and eukaryotic autotrophs.

In the present study the genetic manipulation of tetrapyrrole biosynthesis pathway that governs carbon, nitrogen and sulphur assimilation has given an insight to the co-regulation of these three different but inter-dependent biological processes. In the present study metabolism of chlorophyll, nitrogen and sulphur is genetically manipulated by over-expression or silencing of divinyl reductase (Dvr) and sirohydrochlorin ferrochelatase (SirB) required for carbon, nitrogen and sulphur assimilation.

Genetic manipulation of Sirohydrochlorin ferrochelatase

Higher plant sulphite and nitrite reductases contain siroheme as a prosthetic group. Siroheme is synthesized from uroporphyrinogen III an intermediate of biosynthetic pathway of chl via methylations and ferrochelation reaction catalyzed by uroporphyrinogen III methyltransferase and sirohydrochlorin ferrochelatase. Sirohydrochlorine ferrochelatase is the terminal enzyme of siroheme biosynthesis that is a 2Fe-2S containing enzyme incorporating Fe into the Siroheme structure. Siroheme is required as a prosthetic group in the six-electron reduction processes of both sulphite and nitrite reductases involved into sulphur and nitrogen assimilation respectively.

Sirohydrochlorine ferrochelatase (SirB) is nuclear coded enzyme having transit peptide that helps the enzyme to be targeted to the chloroplast. \( At\text{SirB} \) contains 678 bp coding for precursor protein of 225 amino acids out of which 46 act as N-terminal transit peptide. Chloroplast is also the location of sulphite and nitrite reductase that requires siroheme as a prosthetic group.
The present study reveals that *AtSirB* itself is a light-regulated gene that is present in very small amounts in etiolated tissues and highly up-regulated after 24 h of continuous light exposure. Since siroheme is the prosthetic group of NiR and SiR it is quite likely that their activities are also light regulated.

The *AtSirB* transgenic *Arabidopsis* plants having sense and antisense expression under constitutive 35S promoter were raised. *AtSirB* gene and protein expression substantially increased in sense plants and decreased in *antiSirB* plants.

*AtSirBx* plants were bigger in size and greener in colour as compared to that of WT. Most *antiAtSirB* plants were very pale and could not survive. This could be due to severe silencing of *SirB* resulting in highly impaired NiR and SiR functions and consequent accumulation of toxic metabolites i.e., nitrite and sulphite. The possibility of severe impairment of N and S assimilation also could result in plant death. A few antisense lines survived. However they were smaller than WT. As compared to that of WT, number of lateral roots per plant was higher in *AtSirBx* plants. In antisense plants root length was longer than that of WT. However, number of lateral roots of antisense plants was same as that of WT. The fresh weight and dry weight of *AtSirBx* were more than that of WT whereas in antisense plants were less than that of WT. This could be due to increased nitrogen, sulphur and carbon assimilation in sense plants and their reduced assimilation in antisense plants.

The Chl and carotenoids contents of *AtSirBx* plants were higher than that of WT and in *antiSirB* plants Chl and carotenoids contents were lower than that of WT. As revealed from the increased rate of Pchlide biosynthesis, the Chl biosynthetic potential was higher in *AtSirBx* plants. In antisense plants the Pchlide and consequent Chl biosynthesis potential was lower than that of WT. This was due to increased or decreased protein abundance of Chl biosynthetic enzymes i.e., GluRs, UroD, CPO and ChID in sense and anti-sense plants respectively.

Similar to Chl, the total protein and N contents of mature (3-week-old) *AtSirBx* plants were higher than that of WT. Conversely, the protein contents of *antiSirB* plants declined. This was due to increased or decreased NR and NiR activities of *AtSirB* sense and antisense plants respectively. The increased activity of NR was due to increased gene expression of *Nia2*, that encodes nitrate reductase structural gene, involved in nitrate assimilation. *Nia2* is predominantly expressed in
Arabidopsis. The augmented NiR activity was due to increased NiR gene (Nii) and protein expression in SirB overexpression lines. Therefore, increased activity of NiR was due to increased availability of apoprotein and the prosthetic group siroheme.

In antisense plant, unexpectedly the gene expression of Nia2 coding for NR and Nii coding for NiR increased in SirB antisense lines. In the same vein, the apoprotein expression of NiR also increased. However, the NiR and NR activities declined in antisense plants resulting in reduced total protein contents of antisense plants. The increased apoprotein contents of NiR in antiSirB plants could be due to an adaptive response of plants to synthesize more proteins to bind to reduced amounts of the available cofactor siroheme. Although NiR apoprotein contents increased, due to reduced availability of the cofactor siroheme, the NiR activity declined. The NR activity also declined in antiSirB plants as siroheme is not directly required by NR.

The most pronounced outcome of the present study is the increased protein contents of AtSirBx. The prosthetic group siroheme could be a limiting factor in N assimilation and its improved synthesis in AtSirbx plants could be a crucial factor in the assembly and activity of NiR. Better availability of siroheme increased NiR activity and assimilation of NO3- to NH4+. Increased NH4+ would have stimulated aminoacid and protein synthesis. Increase protein content upregulated photosynthesis resulting in larger phenotype, and higher biomass accumulation in transgenic plants. Stimulation of cofactor synthesis rather than apo-protein accumulation results in improved NUE.

The SirB overexpressors could tolerate N-starvation

As stated above, Arabidopsis plants overexpressing AtSirB had larger phenotype and increased protein contents. To ascertain if these plants could tolerate N starvation, WT and AtSirBx Arabidopsis plants were grown in N deficient media. Due to N deficiency the phenotype of WT plants looked pale-green and in extreme N starvation (0.1N of control), they almost blanched. This was due to reduced Chl accumulation. Under identical growth conditions the AtSirBx plants looked greener than WT and had higher amounts of Chl than that of WT. However, in antiSirB plants the N starvation caused severe phenotypical changes including bleaching even at 0.5N due to severe reduction of their Chl contents.
As compared to that of WT, total protein contents were higher in \textit{AtSirBx} and lower in \textit{antiSirB} plants. Under normal (1N) N level, protein contents of overexpressor were higher and in under-expressors the same were lower than that of WT. In extreme N-starvation conditions, the protein contents of \textit{AtSirBx} plants were much higher than that of WT. Conversely, due to extreme N starvation the antisense plants had a lot lower proteins as compared to optimal growth medium. Decreased protein contents of plants grown in nitrate-deficient media was due to reduced NiR and NR activities. The NiR and NR activities declined in \textit{antiSirB} plants. In N deficient conditions, as compared to that in optimal growth medium (1N), in sense and antisense plants, the NiR activities of \textit{AtSirBx} plants under extreme N starvation, were much higher than that of WT and a lot lower in antisense plants. Similar to their activities, in optimal growth media, \textit{NR} and \textit{NiR} gene expression increased in \textit{AtSirBx} plants. However, in antisense plans their expression was not reduced in optimal growth media. In N-deficient growth media both \textit{NR} and \textit{NiR} expression severely declined in WT and highly reduced in antisense plants. Under identical conditions, their gene expression was only partially declined in \textit{AtSirBx} plants. Results demonstrate that NR and NiR activities well correlate to their gene expression.

\textbf{\textit{AtSirB} Overexpression Overcomes Sulphur Deficiency} \\
Sulphate is taken up and then assimilated to cysteine and reduced in the chloroplast. When plants are starved for sulphur, they activate mechanisms for increasing acquisition from soil. However, when plants cannot acquire enough sulphate, the decreased sulphate uptake leads to reduced assimilation activity and affects many different metabolic processes. Eventually, the limited supplies of sulphur in plants result in decreased plant tissue sulphur content. Decreases in sulphur content results in the inhibition of sulphate assimilation, decreased cysteine methionine contents, reduced chlorophyll, total protein and nitrogen imbalance. Overall, these changes lead to a reduced rate of metabolism and growth.

After 7 days of growth in S-deficient medium (0.1S) the phenotype of WT plants were deficient in Chl and looked pale-green whereas the \textit{AtSirBx} plants accumulated higher amounts of Chl and looked greener than WT. However, in \textit{antiSirB} plants the S starvation caused severe phenotypical changes and the plants became severely blanched due to severe reduction of Chl contents. Carotenoids are
capable of quenching ROS i.e., singlet oxygen. Therefore, although their Chl contents decreased, the carotenoids contents of S-deficient WT, sense and antisense plants increased. However, the percent increase of carotenoids contents in S-starved AtSirB antisense plants was highest suggesting that singlet oxygen could be produced in large amounts in these plants.

In AtSirBx the protein abundance of SiR was substantially higher than that of WT. However, in antisense lines their abundance also increased similar to that of over-expressors. In agreement with that of protein expression, as compared to that of WT, SiR expression substantially increased in AtSirBx plants. In antisense plants SiR message abundance also increased, although to a reduced extent. In the plastid ferredoxin-dependent SiR converts sulphite to sulphide that is used to synthesize cysteine and methionine. Increased availability of S-containing amino acids in AtSirBx plants resulted in the synthesis of larger amounts of proteins than that of WT. Conversely, antisense plants had reduced protein contents probably due to reduced activity of SiR that generated smaller amounts of S-containing aminoacids.

Increased availability of S-containing aminoacids could lead to increased availability of glutathione that could play a positive role in maintaining a reduced atmosphere in the cytosol and cell organelles to positively facilitate metabolic reactions and could protect plants against oxidative stress. The increased S nutrition could result in augmented assembly of Fe-S centers in AtSirBx. This also resulted in increased synthesis of Fe-S containing proteins i.e., Rieske protein required in the cytochrome b/f complex of chloroplasts in AtSirBx plants. Conversely, the Rieske protein declined in antiSirB plants.

**Divinyl Reductase Overexpression Increases Chlorophyll Biosynthesis and Photosynthesis in Arabidopsis thaliana and Brassica juncea**

The AtDvr transgenic of Arabidopsis plants having sense and antisense expression under constitutive 35S promoter was confirmed by PCR of genomic DNA and Southern blot analysis. The semi-quantitive RTPCR and Northern blot analysis revealed that the AtDvr expression substantially increased in AtDvrx plants and decreased in antiDvr plants. In the same vein as compared to that of WT, the protein expression significantly increased in AtDvrx plants and declined in antiDvr plants.
In comparison with WT plants, \textit{AtDvrx} plants were not phenotypically significantly different from that of WT. Among morphological parameters i.e., plant height, rosette diameter, root length, and fresh weight, the latter was partially higher in \textit{AtDvrx} plants. However, as compared to that of WT, in \textit{antiDvr} plants the plant height, rosette diameter, root length, and fresh weight were reduced.

The \textit{AtDvrx} plants had higher Chl and carotenoid contents than that of WT. However, in antisense plants, the Chl contents decreased and therefore had a pale phenotype. Due to preferential loss of Chl b, the Chl a/b ratio increased in antisense plants. This suggests the preferential loss of Chl b enriched light-harvesting antenna complex in antisense plants. Although, Chl contents decreased in antisense plants, their carotenoids contents increased. Pchlide is one important intermediate of Chl biosynthesis pathway. It increased in \textit{AtDvrx} plants that led to increased Chl synthesis in \textit{AtDvrx} plants. Conversely, the Chl biosynthesis potential decreased in \textit{antiDvr} plant leading to reduced Pchlide and Chl contents. Using appropriate equations, the ratio of MV Pchlide/DV Pchlide was calculated. In green sense plants due to overexpression of \textit{AtDvr} the proportion of DV Pchlide decreased and consequently that of MV Pchlide increased in \textit{AtDvrx} plants. In \textit{antiDvr} plants due to reduced Dvr reaction the relative the proportion of DV Pchlide increased and consequently that of MV Pchlide decreased.

Increased dry matter accumulation in \textit{AtSirBx} plants and decreased biomass of \textit{antiDvr} plants suggest that their photosynthesis efficiencies would be higher or lower than that of WT. The photosynthetic potential of WT, \textit{AtDvrx}, \textit{antiDvr} plants grown in pots was probed using Chl a fluorescence as a signature. The Fv/Fm ratio, an estimate of the maximum portion of absorbed quanta used in PSI! reaction centers by dark-adapted leaves, was not altered in transgenic lines. However, as compared to WT, the ETR (µmoles electrons m\(^{-2}\) s\(^{-1}\)) was higher in \textit{AtDvrx} plants at saturating light intensities. In \textit{antiDvr} plants the ETR substantially declined. This suggest that efficient conversion of DV Chl biosynthesis intermediates to monovinyl forms favours the greening process leading to increased photosynthesis and dry matter accumulation. On the contrary the reduced Dvr function that preferentially favours the formation of divinyl intermediates reduces the Chl contents, photosynthetic efficiency and dry matter accumulation.
**AtDvr overexpression in *Brassica juncea***

*AtDvr* profusely expressed in Brassica sense plants. The *Dvr* overexpression led to increased Chl, carotenoids and total protein contents. Increased Chl content of *DvrBx* plants was due to their increased biosynthetic potential. As revealed from increased protochlorophyllide biosynthesis of the transformants. Similar to *Arabidopsis*, green WT *Brassica* plants were predominantly divinyl that accumulated Pchlide in divinyl form. The *DvrBx* plants had increased conversion of DV Pchlide to MV Pchlide resulting in reduced accumulation of DV Pchlide with concomitant increase of MV Pchlide. However, etiolated tissues of Brassica was predominantly monovinyl that accumulated of Pchlide in MV form. In *DvrBx* due to efficient conversion of residual DV Pchlide to MV Pchlide, the proportion of DV Pchlide decreased and MV Pchlide increased. The differential proportion of MV and DV Pchlide in etiolated and green tissues of WT plants could be due to photoregulation of the synthesis of DV Pchlide and slow but steady conversion of DV Pchlide to MV Pchlide during prolonged period of darkness. This is evident from 3 h dark incubation of green leaf discs in water or with ALA. During dark incubation the DV Pchlide was converted to MV Pchlide that led to reduction in the proportion of DV Pchlide. In ALA-treated green leaf discs although high amounts of DV Pchlide were synthesized they were also converted to the MV form in *DvrBx* plants.

It is apparent that increased presence of MV form and decreased presence of DV form of Chl biosynthesis intermediate in the *DvrBx* plants of Brassica led to increased Chl biosynthesis and their photosynthesis potential. This is evident from increased ETR and decreased non-photochemical quenching of Chl a fluorescence suggesting that the release of excitation pressure as heat decreased in the sense plants.

MVChl is very essential for efficient energy capture in higher plants. DVChl cannot efficiently substitute for MVChl in higher plants. DV Pchlide is photo-transformed to DV Chlide by the light-dependent protochlorophyllidr oxidoreductase. DV Chlide is converted to MV Chlide by Dvr. The conversion of DV Chlide to MV Chlide is very efficient and faster than that of DV Pchlide to MV Pchlide. Accumulation of DV Chlide in light could be harmful to the plant and therefore it is converted to MV Chlide very fast. Therefore, in antisense plants and *dvr* mutants that accumulate large amounts of DV Chlide probably have light-induced injury to them.
Summary

via generation of ROS. This could result in reduced Chl content, photosynthesis and plant growth. As DV Pchlide accumulates in nature in etiolated and in night time of green Cucumber and several other plants, it is proposed that it does not generate ROS. It is concluded that efficient conversion of DV Pchlide to MV Pchlide and DV Chlide to MV Chlide favours chlorophyll biosynthesis, chloroplast development and photosynthesis.